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ammonium (TEA) suggests that these agents occupy structurally homologous binding sites close to or within the mouth of the channel pore (20). Guy and Conti (27) and Hille (20) suggested an alignment of amino acid sequences where the Glu³⁷⁶ involved in TTX and STX binding in Na⁺ channels is one to two positions away from the critical residue at position 449 in K⁺ channels, a major site in charybdotoxin and TEA binding. Our results with the Cys³⁷⁴ → Tyr and the Arg³⁷⁷ → Asn mutants demonstrate that residues 374 to 377 are essential for binding of TTX and STX and Cl⁻, which would align with residues 444 to 447 of the Shaker K⁺ channel (20, 27). This would place the TTX and STX receptor about three-eighths to three-quarters of the way into the pore, which is inconsistent with the observed lack of voltage dependence for TTX and STX binding. Identification of the residue at position 374 as critical to high-affinity toxin binding reveals the structural difference that distinguishes TTX-R and TTX-S Na⁺ channel isoforms and explains the high-affinity divalent cation blockage of the RHI and the competition of divalent ions and toxin for binding. The position of this residue may require revision of the Na⁺ channel pore structure suggested by homology with the K⁺ channel.

Note added in proof: Cys³⁷⁴ → Phe has the same properties as Cys³⁷⁴ → Tyr.

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Identification of Heregulin, a Specific Activator of p185^{erbB2}

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The proto-oncogene designated *erbB2* or HER2 encodes a 185-kilodalton transmembrane tyrosine kinase (p185^{erbB2}), whose overexpression has been correlated with a poor prognosis in several human malignancies. A 45-kilodalton protein heregulin- α (HRG- α) that specifically induced phosphorylation of p185^{erbB2} was purified from the conditioned medium of a human breast tumor cell line. Several complementary DNA clones encoding related HRGs were identified, all of which are similar to proteins in the epidermal growth factor family. Scatchard analysis of the binding of recombinant HRG to a breast tumor cell line expressing p185^{erbB2} showed a single high affinity binding site [dissociation constant (K_d) = 105 \pm 15 picomolar]. Heregulin transcripts were identified in several normal tissues and cancer cell lines. The HRGs may represent the natural ligands for p185^{erbB2}.

The p185^{erbB2} protein is a 185-kD transmembrane tyrosine kinase encoded by the *erbB2* proto-oncogene (1) that is similar to the epidermal growth factor (EGF) receptor (2) and the HER3, or c-*erbB3*, protein (3). Both p185^{erbB2} and the EGF receptor are associated with certain human malignan-

cies (4). In particular, overexpression of p185^{erbB2} correlates with a poor prognosis in breast, ovarian, gastric, and endometrial cancers and non-small cell lung adenocarcinoma (5). EGF and transforming growth factor- α (TGF- α), which are ligands for the EGF receptor, clearly promote cell growth and transformation (1, 6). However, a similar dependence on a ligand for growth or transformation in cells expressing p185^{erbB2} has not been established. Neither EGF nor TGF- α binds to or activates p185^{erbB2} (7).

Evidence that p185^{erbB2} may respond to exogenous ligands includes observations

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HRG- α was detected in conditioned medium of MDA-MB-231 human breast carcinoma cells as a factor that stimulated the tyrosine kinase activity of p185^{hbk} in MCF-7 breast tumor cells (12) (Fig. 1). We purified the 45-kD protein by chromatography on heparin Sepharose and cation exchange columns and by reversed-phase chromatography. Amino acid sequence data derived from proteolytic digestion products (13) enabled the design of a deoxy-oligonucleotide probe that was used to

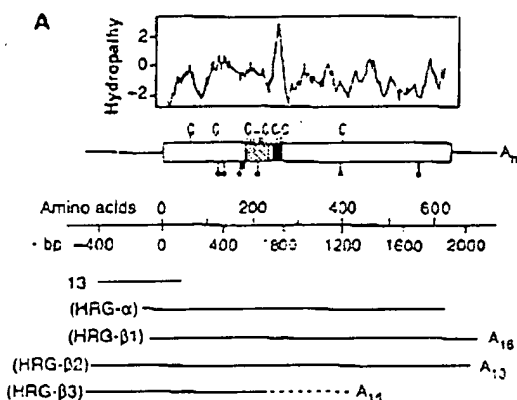


Fig. 2. Amino acid sequence of the neregulins and their relation to EGF family members. (A) HRG cDNAs. Hydropathy profile indicating the position of a hydrophobic domain at positions 243 to 265 (top). The coding area is boxed and cysteines are indicated (bottom). Hatching, six-cysteine EGF-like structural unit; filled box, hydrophobic segment. (♢) six potential asparagine-linked glycosylation sites (29); small filled box, area for possible O-linked glycosylation (30). The lengths and positions of clones 13, proHRG- α , proHRG- β 1, proHRG- β 2, and proHRG- β 3 are shown with poly(A) tails designated (A_n). The dashed line of proHRG- β 3 is a non-homologous 3' untranslated sequence. (B) Amino acid sequences of the proHRGs. Amino acid sequence and alignment of three proHRG forms relative to proHRG- α . Amino acid numbers, indicated on the left, are based on proHRG- α . Shaded area, amino acids within the divergent region of the HRGs that occur in at least two forms; asterisks, COOH-terminal amino acids; overline, the 23-amino acid hydrophobic region; double dots, possible sites of glycosaminoglycan addition (31); triple dots, possible sites of N-linked glycosylation; double overline, area for possible O-linked glycosylation; underline, a potential nuclear targeting signal; triangles, two sites of single amino acid substitutions. Gln 38 is replaced by Arg in proHRG- β 1, - β 2, and - β 3 and Met is replaced by Lys in proHRG- β 2 at position 463. (C) Alignment of HRGs with human EGF family members. Shown are the EGF-like structural units of the indicated molecules. HRG sequences begin with amino acid 175 of the proforms, other proteins are numbered relative to amino acid 1 of their mature forms. Amino acids strictly con-



served are shaded. Dashes, gaps in the sequence inserted for best alignment; overlines, known or putative hydrophobic domains; arrows, positions of proposed (proHRG) or known (others) COOH-terminal processing; asterisk, COOH-terminus of proHRG-83.

REPORTS

screen a λ gt10 cDNA library constructed with mRNA from the MDA-MB-231 cells (14). One hybridizing clone, which appeared to encode a precursor of HRG- α (proHRG- α), was isolated and sequenced (GenBank accession numbers M94165, M94166, M94167, and M94168). This

clone (Fig. 2A) encodes a reading frame that is open at both the 5' and 3' ends. Related coding sequences were discovered in other λ gt10 libraries from MDA-MB-231 cells (14) as cDNAs that encode the NH₂- and COOH-termini of proHRG- α (Fig. 2A). The cDNAs encode proteins of 640,

645, 637, and 231 amino acids, which we have termed proHRG- α , proHRG- β 1, proHRG- β 2, and proHRG- β 3, respectively. The initiating methionine was assigned on the basis of an in-frame stop codon 444 bases upstream. Because a termination signal (TAA) exists at identical positions in both proHRG- β 1 and proHRG- β 2 cDNAs, a stop was assigned at a corresponding site in the sequence of proHRG- α . The four HRGs are distinguished by a variable region of 19 to 29 amino acids beginning at position 212 (Fig. 2B). The predicted amino acid sequences of proHRG- β 1, proHRG- β 2, and proHRG- β 3 diverge from that of proHRG- α at this point and for the next 18 residues (through position 230) they share a common sequence before diverging further and losing similarity to one another. After residue 230, proHRG- β 1 continues for nine more amino acids and proHRG- β 2 continues for a single amino acid before both resume identity with proHRG- α at proHRG- α position 235. The proHRG- β 3 cDNA encodes 11 residues beyond position

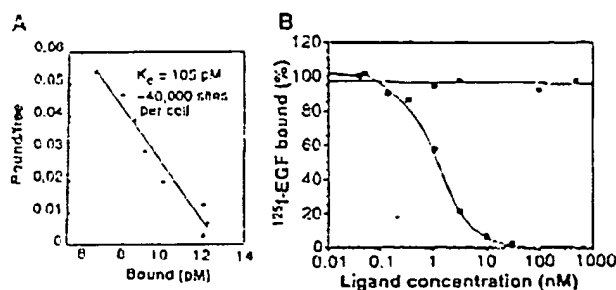
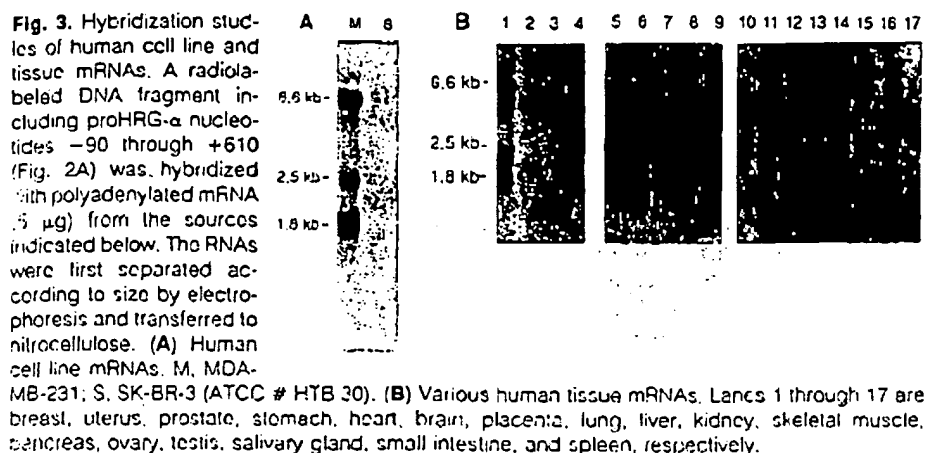


Fig. 4. Specificity of interaction of HRGs with p185^{HER2}. (A) Scatchard analysis of ¹²⁵I-labeled HRG- β 1₁₇₇₋₂₄₁ binding to MCF-7 cells. *Escherichia coli*-expressed, truncated rHRG- β 1₁₇₇₋₂₄₁ (24) was labeled with ¹²⁵I-Bolton-Hunter reagent (ICN) (32) and purified by reversed-phase HPLC (specific activity, 150 μ Ci/ μ g). MCF-7 cells (ATCC # HTB 22) were incubated in medium with various concentrations of unlabeled rHRG- β 1₁₇₇₋₂₄₁ and a constant amount of ¹²⁵I-labeled rHRG- β 1₁₇₇₋₂₄₁ for 16 hours at 4°C. Unbound ligand was removed, and cells were washed with ice-cold medium. The amount of radioactivity bound was determined after solubilizing the cells with 0.1 N NaOH containing SDS (0.1%). Binding data were analyzed using a nonlinear regression program (33). (B) Competitive inhibition of binding of ¹²⁵I-labeled EGF to A431

cells (ATCC # CRL 1555). Cells were incubated with various concentrations of EGF (squares) or rHRG- β 1₁₇₇₋₂₄₁ (circles) as described above but with ¹²⁵I-labeled EGF (specific activity, 200 μ Ci/ μ g). (C) Cross-linking of ¹²⁵I-labeled rHRG- β 1₁₇₇₋₂₄₁ to cell lines expressing p185^{HER2}. SK-BR-3, MDA-MB-453 (ATCC # HTB 131), or MCF-7 cells (2.0×10^5) were suspended in Hank's balanced salts (Gibco-BRL) and incubated with ¹²⁵I-labeled rHRG- β 1₁₇₇₋₂₄₁ (10^6 cpm) in the absence (–) or presence (+) of unlabeled rHRG- β 1₁₇₇₋₂₄₁ (100 nM) for 30 min at 22°C. Eris(sulfosuccinimidyl) suberate (Pierce) was added to the cell suspensions (final concentration, 1 mM) and the incubations were continued for 30 min. The cells were washed with Tris-buffered saline (TBS) and dissolved in SDS sample buffer. Samples were run on a polyacrylamide gel (7%) and bands were visualized by autoradiography. Molecular size standards are in kilodaltons. (D) Cross-linking of ¹²⁵I-labeled rHRG- β 1₁₇₇₋₂₄₁ to MDA-MB-453 cells and immunoprecipitation of the complex with antiserum to p185^{HER2}. ¹²⁵I-labeled rHRG- β 1₁₇₇₋₂₄₁ was cross-linked to MDA-MB-453 cells as described in (C) except that after the TBS wash, the cells were lysed in TBS containing Triton X-100 (0.5%). Immunoprecipitations (IP) were performed on portions of each lysate with either the guinea pig antiserum to the extracellular domain of p185^{HER2} or normal guinea pig serum as a control. Samples were prepared in SDS sample buffer, run on a polyacrylamide gel (5.5%), and visualized by autoradiography. Lane 1, cell lysate in the absence (–) of unlabeled HRG; lane 2, cell lysate in the presence (+) of HRG (100 nM); lane 3, same as lane 1 but immunoprecipitated with anti-p185^{HER2}; lane 4, same as lane 1 but with normal goat serum; lane 5, same as lane 2 but immunoprecipitated with anti-p185^{HER2}. (E) Cross-linking of ¹²⁵I-labeled EGF to MDA-MB-468 cells (ATCC # HTB 132). Cells were suspended in Hank's balanced salts and incubated with ¹²⁵I-labeled EGF (Amersham). Lane 1, cell lysate in the absence (–) of unlabeled EGF; lane 2, cell lysate in the presence (+) of EGF (100 nM); and lane 3, cell lysate in the presence (+) of 100 nM rHRG- β 1₁₇₇₋₂₄₁. Cross-linking was performed as described in (C). The cells were dissolved in SDS sample buffer and run on a polyacrylamide gel (5.5%). Bands were visualized by autoradiography.

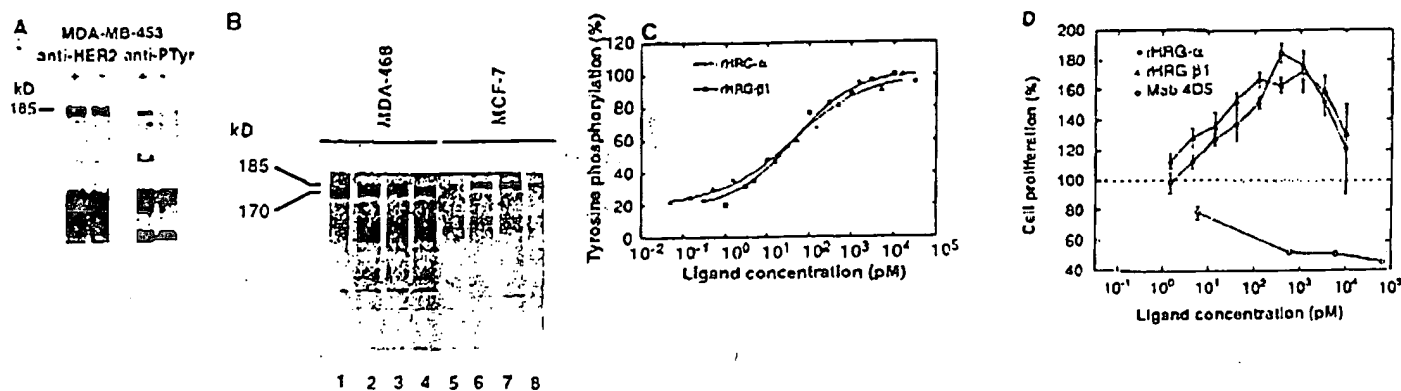


Fig. 5. Specificity and mitogenic activity of heregulins. **(A)** Immunoprecipitation of p185^{erbB2} after stimulation of MDA-MB-453 cells. Cells (2.0×10^6) were suspended in 50 mM Hepes (pH 7.4) buffer containing 150 mM NaCl, 2 mM MgCl₂, 1 mM MnCl₂, and 1 mM EGTA and incubated in the presence (+) or absence (-) of rHRG-β1 (4.0 nM) (24) for 15 min at 22°C. Cells were lysed in the same buffer containing Triton X-100 (1.0%), 200 μM Na₃VO₄, and 10 mM EDTA. The p185^{erbB2} was immunoprecipitated with guinea pig antiserum to the extracellular domain of p185^{erbB2}. Blots were probed with antiserum (αHER2) to a synthetic peptide corresponding to a COOH-terminal region of p185^{erbB2} (34) or with a monoclonal antibody to phosphotyrosine (12). Bands at the bottom of the gel are due to the reaction of secondary antibodies with the antibodies used in the immunoprecipitation. **(B)** Stimulation of tyrosine phosphorylation in MDA-MB-468 and MCF-7 cells. Cells were treated with various concentrations of EGF, rHRG-α, or rHRG-β1 and the increase in tyrosine phosphorylation of the EGF receptor (p170) and p185^{erbB2} was quantitated. Lanes 1 to 4, MDA-MB-468 cells; lanes 5 to 8, MCF-7 cells. Lane 1, human recombinant EGF (1 nM) (Upstate Biotechnology); lane 2, rHRG-α (10 nM); lane 3, rHRG-β1 (10 nM); lane 4, no ligand; lane 5, EGF (10 nM); lane 6, rHRG-α (1 nM); lane 7, rHRG-β1 (1 nM); and lane 8, no ligand. **(C)** Stimulation of tyrosine phosphorylation by rHRG-α and rHRG-β1. MCF-7 cells were incubated with various concentrations of rHRGs, and the increase in tyrosine phosphorylation of p185^{erbB2} quantitated as described (12). The data were fit to a four-parameter nonlinear least squares equation. EC₅₀'s for both rHRG-α and rHRG-β were ~40 pM. **(D)** Stimulation of proliferation of SK-BR-3 cells. Cells (10^4 cells per well) were plated in 96-well microtiter plates and incubated with rHRGs or the cytostatic monoclonal antibody 4D5 (8) for three days before quantitation with crystal violet (8). Quadruplicate wells were tested at each sample concentration. Half-maximal stimulation was observed at ~20 pM.

230 before a stop codon is encountered.

Amino acid hydropathy analysis (Fig. 2A) of proHRG-α, -β1, and -β2 revealed a 23-amino acid hydrophobic region including amino acids 243 to 265 (Fig. 2) that may be a transmembrane domain or internal signal sequence. This suggests that the mature HRG-α, -β1, and -β2 molecules of 45 kD whose sequences are found on the NH₂-terminal side of this region may be derived from a transmembrane-anchored precursor molecule. The proHRG-β3 protein does not contain this hydrophobic region and is predicted not to be membrane-bound. Unlike typical signal sequences the NH₂-terminal amino acids of these proteins are not hydrophobic. Instead, the first 23 amino acids are dominated by charged residues and contain a sequence (GKKKER; residues 13 to 18) that closely resembles the consensus sequence motif for nuclear targeting (15).

The NH₂-terminus determined for HRG-α (13) begins with serine 2 encoded by the cDNA. Analysis of COOH-terminal peptides (13) of the mature HRGs indicated that none of the COOH-termini extended beyond HRG-α position 241 (Fig. 2C). Thus, the mature HRGs probably range in size from 228 to 241 amino acids with a predicted molecular size of ~26 kD. Post-translational modification occurring at predicted glycosylation sites could account for the difference between the predicted and observed (~45 kD by SDS-PAGE under reducing conditions)

molecular sizes of the HRGs.

The HRGs, with the exception of HRG-β3, each contain twelve cysteine residues (Fig. 2), 6 of which are clustered within a 40-amino acid segment adjacent to the putative transmembrane region. The characteristic number and relative positions of these cysteines, as well as the strict conservation of two glycines and an arginine residue within this region (Fig. 2C), establish the HRGs as members of the EGF family (16). Between the first and sixth cysteines the HRGs are most similar (45%) to heparin-binding EGF-like growth factor (HB-EGF) (17). In this same region they are 35% identical to amphiregulin (AR) (18), 32% identical to TGF-α (19), and 27% identical to EGF (16). Outside of this EGF motif there is little similarity between HRGs and other members of the EGF family. EGF, AR, HB-EGF, and TGF-α are all derived from membrane-anchored precursors that are cleaved on both sides of the EGF structural unit to yield 50- to 84-amino acid mature proteins (16-19). Like the EGF family members, the HRGs appear to be derived from a membrane-bound precursor but require only a single cleavage, COOH-terminal to the cysteine cluster, to produce mature proteins.

The expression of HRG mRNA in two breast carcinoma cell lines was studied by Northern (RNA) blot analysis with a cDNA probe encoding the NH₂-terminal portion of proHRG-α. Three major bands, representing transcripts of 6.6, 2.5, and 1.8

kb, were detected in mRNA from MDA-MB-231 cells (Fig. 3A). No HRG mRNA was detected in the SK-BR-3 cell line, which overexpresses p185^{erbB2}.

The various cDNA clones were mapped to the MDA-MB-231 transcripts by hybridization with a panel of synthetic oligonucleotide probes (20). The NH₂-terminal region common to all four HRG subtypes hybridized with all three transcripts. Oligonucleotides corresponding to the sequence of HRG-α hybridized predominantly with the 6.6- and 2.5-kb transcripts, and oligonucleotides corresponding to the sequence of HRG-β hybridized more strongly with the 1.8-kb transcript. The common hydrophobic and COOH-terminal encoding regions of HRG-α, -β1, and -β2 mRNAs hybridized with only the 6.6-kb and 2.5-kb transcripts, whereas the divergent 3' region of HRG-β3 hybridized only with the 1.8-kb message (21).

Various human tissues were also examined for the presence of HRG mRNA (Fig. 3B). Transcripts were found in breast, ovary, testis, prostate, heart, skeletal muscle, lung, liver, kidney, salivary gland, small intestine, brain, and spleen but not in stomach, pancreas, uterus, or placenta. Although most of these tissues contained the same three classes of transcripts as the MDA-MB-231 cells, only the 6.6-kb message was observed in heart and skeletal muscle. In brain a single transcript of 2.2 kb was detected and in testis the 6.6-kb transcript was present along with others of 2.2, 1.9, and 1.5 kb. The tissue-specific expres-

sion pattern observed for HRG differed from that of p185^{erbB2}. Adult liver, spleen, and brain contain HRG but not p185^{erbB2} transcripts (22), whereas stomach, pancreas, uterus, and placenta contain p185^{erbB2} transcripts (23) but lack HRG mRNA.

To characterize physical and functional properties of the HRGs, recombinant proteins were produced. Recombinant HRG- α (rHRG- α) and recombinant HRG- β 1 (rHRG- β 1) representing mature (~45 kD) processed forms of proHRGs were purified from the conditioned medium of mammalian cells transfected with full-length cDNA clones (24). Truncated versions of rHRG- α (rHRG- α ₁₇₇₋₂₄₁) and rHRG- β 1 (rHRG- β 1₁₇₇₋₂₄₁) were produced in *Escherichia coli* (24). Direct binding of rHRG- β 1₁₇₇₋₂₄₁ to p185^{erbB2} was demonstrated in several ways. Scatchard analysis showed a single class of high-affinity binding sites (K_d of 105 ± 15 pM) on the surface of MCF-7 cells (Fig. 4A), a cell line that expresses low levels of p185^{erbB2} (25). Similar K_d 's were observed with MDA-MB-453 cells and SK-BR-3 cells (26). Whereas unlabeled EGF fully competed with ¹²⁵I-labeled EGF for binding to A431 human epidermoid carcinoma cells [2×10^6 receptors per cell (27)], rHRG- β 1₁₇₇₋₂₄₁ (at concentrations up to 500 nM) failed to compete (Fig. 4B), indicating that rHRG- β 1₁₇₇₋₂₄₁ does not bind to the EGF receptor. Cross-linking of ¹²⁵I-labeled rHRG- β 1₁₇₇₋₂₄₁ to receptors on the surface of SK-BR-3 cells, MDA-MB-453 cells, and MCF-7 cells, which express high, medium, and low amounts of p185^{erbB2}, respectively (25), revealed an ~190-kD complex, consistent with the size expected of a complex between p185^{erbB2} and the rHRG- β 1₁₇₇₋₂₄₁ protein [7.9 kD (26)] (Fig. 4C). The amount of this complex was proportional to the amount of p185^{erbB2} expression on the various cell lines examined. Because of the small size of rHRG- β 1₁₇₇₋₂₄₁, it is difficult to determine the precise stoichiometry of the ~190-kD complex. As has been observed with EGF and the EGF receptor (28) (Fig. 4E) other larger complexes were present that may be oligomeric forms of cross-linked p185^{erbB2} and rHRG- β 1₁₇₇₋₂₄₁. These complexes were eliminated when unlabeled rHRG- β 1₁₇₇₋₂₄₁ was added to the cells before cross-linking. The identity of the cross-linked material was confirmed by immunoprecipitation. Polyclonal antisera from guinea pig to the extracellular domain of p185^{erbB2} precipitated cross-linked complexes from MDA-MB-453 cells that comigrated during SDS-PAGE under reducing conditions with cross-linked complexes that had not been immunoprecipitated (Fig. 4D). Irrelevant guinea pig antisera yielded no complexes after precipitation. Cross-linking of ¹²⁵I-labeled EGF to EGF receptors on the surface of MDA-MB-468 cells was abolished

by addition of excess unlabeled EGF and unaffected by addition of rHRG- β 1₁₇₇₋₂₄₁ (Fig. 4E), thus further substantiating that rHRG- β 1₁₇₇₋₂₄₁ does not compete for EGF binding to the EGF receptor.

We immunoprecipitated p185^{erbB2} from MDA-MB-453 cells (which contain moderate amounts of p185^{erbB2} but no detectable EGF receptor) (25) and detected the protein by immunoblotting with antibodies to phosphotyrosine. The p185^{erbB2} protein was phosphorylated after treatment with ~45-kD rHRG- β 1 purified from mammalian cells (Fig. 5A), or similarly derived ~45-kD rHRG- α (26). The same result was obtained with MCF-7 cells, but a less intense signal was observed (26). Whereas both ~45-kD rHRG- α and ~45-kD rHRG- β 1 stimulated p185^{erbB2} phosphorylation in MCF-7 cells, neither protein activated phosphorylation of the EGF receptor in MDA-MB-468 cells (which contain large amounts of the EGF receptor) (25) (Fig. 5B). This result is consistent with the fact that rHRG- β 1₁₇₇₋₂₄₁ bound directly to p185^{erbB2} (Fig. 4, A and D) and did not compete for binding to the EGF receptor (Fig. 4, B and E).

The relative ability of ~45-kD rHRG- α and - β 1 to induce phosphorylation of p185^{erbB2} was evaluated. Both proteins stimulated phosphorylation of p185^{erbB2} on MCF-7 cells with a medium effective concentration (EC_{50}) of ~40 pM (Fig. 5C). Both rHRG- α and - β 1 stimulated the growth of SK-BR-3 cells with an EC_{50} of ~20 pM (Fig. 5D). Optimal stimulation by either molecule was observed at ~400 pM; higher concentrations up to 10 nM were less effective in stimulating cell growth. A p185^{erbB2} ligand has been described that inhibits the growth of SK-BR-3 cells (9).

The heregulin family described here shows the characteristics expected of a ligand that interacts specifically with p185^{erbB2}. The molecular properties of the heregulins are different from those of other ligands that have been described (9-11) in that they have a different size, they stimulate rather than inhibit the proliferation of breast cancer cells in monolayer culture, and they increase tyrosine phosphorylation of p185^{erbB2} but not of the EGF receptor.

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12. Heregulin was purified from medium conditioned by MDA-MB-231 breast cancer cells (ATCC # HTB 26) grown on Percell BioLytics microcarrier beads (Hyclone Labs). The medium (10 liters) was concentrated ~25-fold by filtration through a membrane (10-kD cutoff) (Millipore) and clarified by centrifugation and filtration through a filter (0.22 μ m). The filtrate was applied to a heparin Sepharose column (Pharmacia) and the proteins were eluted with steps of 0.3, 0.6, and 0.9 M NaCl in phosphate-buffered saline. Activity in the various chromatographic fractions was measured by quantitating the increase in tyrosine phosphorylation of p185^{erbB2} in MCF-7 breast tumor cells (ATCC # HTB 22). MCF-7 cells were plated in 24-well Costar plates in F12 (50%) Dulbecco's minimum essential medium (50%) containing serum (10%) (10⁵ cells per well), and allowed to attach for at least 24 hours. Prior to assay, cells were transferred into medium without serum for a minimum of 1 hour. Column fractions (10 to 100 μ l) were incubated for 30 min. at 37°C. Supernatants were then aspirated and the reaction was stopped by the addition of SDS-PAGE sample buffer (100 μ l). Samples were heated for 5 min. at 100°C, and portions (10 to 15 μ l) were applied to a 10% glycine gel (4 to 20%) (Novex). After electrophoresis, proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane and then blocked with bovine serum albumin (5%) in Tris-buffered saline containing Tween-20 (0.05%) (TBST). Blots were probed with a monoclonal antibody (1:1000 dilution) to phosphotyrosine (Upstate Biotechnology) for a minimum of 1 hour at room temperature. Blots were washed with TBST, probed with an antibody to mouse immunoglobulin G conjugated to alkaline phosphatase (Promega) (diluted 1:7500) for a minimum of 30 min at room temperature. Reactive bands were visualized with 5-bromo-4-chloro-3-indenyl-1-phosphate and nitro-blue tetrazolium. Immunoblots were scanned with a Scan Jet Plus (Hewlett-Packard) densitometer. Signal intensities for unstimulated MCF-7 cells were 20 to 30 units. Fully stimulated p185^{erbB2} yielded signals of 180 to 200 units. The 0.6 M NaCl pool, which contained most of the activity, was applied to a polyaspartic acid (PolyLC) column equilibrated in 17 mM sodium phosphate (pH 6.8) containing ethanol (30%). A linear gradient from 0.3 M to 0.6 M NaCl in the equilibration buffer was used to elute bound proteins. A peak of activity (at ~0.45 M NaCl) was further fractionated on a C4 reversed-phase column (SynChropak RP-4) equilibrated in buffer containing TFA (0.1%) and acetonitrile (15%). Proteins were eluted from this column with an acetonitrile gradient from 25 to 40% over 60 min. Fractions (1 ml) were collected, assayed for activity (Fig. 1E), and analyzed by SDS-PAGE on Tris-glycine gels (4-20%, Novex).
13. HPLC-purified HRG- α was digested with lysine C in SDS (0.1%), 10 mM dithiothreitol, 0.1 M NH₄HCO₃ (pH 8.0) for 20 hours at 37°C and the resultant fragments were resolved on a SynChrom C4 column (4000 Å, 0.2 by 10 cm). The column was equilibrated in 0.1% TFA and eluted with a 1-propanol gradient in 0.1% TFA [W. J. Henzel, J. T. Stults, C. Hsu, D. W. Aswad, *J. Biol. Chem.* 264, 15905 (1989)]. Peaks from the chromatographic

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